Original article

PSEUDOMONAS AERUGINOSA IN PATIENTS WITH c.1652G›A (G551D) -CFTR TREATED WITH IVACAFTOR - CHANGES IN MICROBIOLOGICAL PARAMETERS

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Running Title: Does ivacaftor change microbiological parameters *in vivo*?

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SUMMARY

What is known and objective: The CFTR potentiator, Ivacaftor (IVA), has been widely used in the treatment of cystic fibrosis (CF) patients with the G551D mutation. To date, there has been limited information on the microbiological status of patients on this therapy and no data on the effect (if any) on the *in vivo* antibiotic susceptibility of *Pseudomonas aeruginosa* isolated from patients on therapy. Whilst IVA intervention is not designed *per se* as anti-infective, the effect (if any) of this molecule to CF patients' microbiological status merits careful monitoring. Therefore, it was the aim of this observational study to examine the effect in patients, both before and after commencement of IVA therapy, on several commonly reported microbiological markers in CF patients, including (i) bacterial density, (ii) frequency (rate) of isolation of bacterial pathogens, particularly *Pseudomonas aeruginosa* and (iii) antimicrobial susceptibility of these isolates to commonly prescribed oral and iv antibiotics. In addition, we wished to examine the requirements for these antibiotics in CF patients, before and after commencement of IVA therapy.

Methods: Archived data from 15 adult patients with the c.1652G›A (G551D) mutation were followed from two years pre-IVA therapy to two years after commencement of IVA therapy. The microbiological parameters examined included (i) oral antibiotic courses taken, (ii) intravenous (iv) antibiotic courses taken, (iii) rate of isolation of non-mucoid Pseudomonas aeruginosa (NM-PA) and mucoid *Pseudomonas aeruginosa* (M-PA), (iv) density of NM-PA and M-PA and (v) antimicrobial susceptibility of NM-PA and M-PA to 11 antibiotics [aminoglycosides, beta-lactams, polymyxin and fluoroquinolone]

Results and discussion: Following commencement of IVA therapy, patients required less iv antibiotic courses but no change in number of oral antibiotics courses. There was significant reduction in both the rate of isolation and density of M-PA ($p=0.02$; $p=0.006$, respectively). In contrast, there was no significant reduction in both the rate of isolation and density of NM-PA ($p=0.90$; $p=0.07$, respectively).

Antimicrobial susceptibility in NM-PA and M-PA was not significantly reduced within any of the antibiotics classes or individual antibiotics examined. Increased susceptibility was noted in the beta-lactam class for NM-PA and M-PA, in particular with ceftazidime.

What is new and conclusion: Overall, (i) the requirement for less iv antibiotic therapy, (ii) a reduction in the rate and density of M-PA, and (iii) no reduction in antibiotic susceptibility, indicates that microbiological parameters with patients on IVA therapy were not detrimentally affected.

***What is known and objective*:**

The dominant feature of cystic fibrosis (CF)-related disease is the deterioration in patients' lung function due to the chronic presence of bacterial pathogens, particularly *Pseudomonas aeruginosa*. Any change in the status of the patient's microbiology may have a significant effect in clinical outcome, in either a positive or negative manner. Outside of pulmonary exacerbation, the relative microbiological stability of the cystic fibrosis (CF) lung is a fine equilibrium of multiple factors. In microbiological terms, these include the presence/absence of bacterial pathogens, numbers of organisms present, antibiotic resistance, carriage/expression of bacterial virulence determinants, relative phase of bacterial growth (e.g. presence of senescent cells/persisters), host/environmental stress responses and relative nutritional/starvation status of bacterial pathogens, iron sequestration and competition and the dynamic flux from the co-habiting microbiome of the lung. Several other host (patient) related factors can also contribute to this instability in this two host [patient & bacterium] system.

In microbiological terms, what is not well understood is the sequencing and interactions of these factors, when they occur together in a particular pattern, what precipitates the tipping of the relatively stable CF lung into a pulmonary exacerbation or alternatively the interactions which drive the CF lung to a more stable situation, thereby reducing infection and exacerbations. In addition, the relative contribution and interaction of the human host undoubtedly plays a significant part in driving this stable equilibrium to a state of relative instability and hence onwards to a potential pulmonary exacerbation.

The 21st century is witnessing the development of many new pharmacological interventions in CF, in order to ameliorate the effects of cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction and thus improve patient outcomes. One such intervention is the use of CFTR correctors/potentiators, as an effective intervention, which has revolutionized CF care in those patients with a genetic profile of their alleles which are predicted to benefit from such interventions.[1]

Ivacaftor [N-(2,4-Di-tert-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide] (VX-770; Kalydeco®) [IVA] is an orally bioavailable CFTR-potentiator molecule, that is designed to increase the time that activated CFTR channels at the cell surface remain open, in CF patients with specific mutations, including G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, or S549R. IVA therapy with genetically appropriate CF patients has been shown to improve lung function, as well as improve(i) risk of pulmonary exacerbations,(ii) patient-reported respiratory symptoms, (iii) weight, (iv) concentration of sweat chloride [1] and (v) quality of life.[2]

Whilst IVA intervention is not designed *per se* as anti-infective, the effect (if any) of this molecule to CF patients' microbiological status merits careful monitoring. Therefore, it was the aim of this study to examine the effect in patients, both before and after commencement of IVA therapy, on several commonly reported microbiological markers in CF patients, including (i) bacterial density, (ii) frequency (rate) of isolation of bacterial pathogens, particularly *Pseudomonas aeruginosa* and (iii) antimicrobial susceptibility of these isolates to commonly prescribed oral and iv antibiotics. In addition, we wished to examine the requirements for these antibiotics in CF patients, before and after commencement of IVA therapy.

Materials and methods

Patient population

A retrospective analysis was conducted on 15 adult patients, who were receiving therapeutic treatment for their cystic fibrosis with oral ivacaftor. Each patient had a confirmed diagnosis of cystic fibrosis, with at least one copy of the G551D mutation. Patients had an age range of 16-43 years and there were nine males (age range: 16 – 43years) and seven females (age range: 18-32 years). Male patients treatment duration ranged from 18-30 months, with a mean treatment duration of 24 months and female patients treatment durations ranged from 18-29 months, with a mean treatment duration of 23.4 months.

Antibiotic treatment and microbiological parameters analysed

Each parameter was examined (i) two years prior to commencement of ivacaftor therapy and (ii). two years post ivacaftor therapy.

Archived microbiological data was analysed from the patients' clinical microbiology record file, with particular reference to (i). total *Pseudomonas aeruginosa* (PA), (ii). non-mucoid (NM) PA and (iii). Mucoid (M) PA. The specific microbiological parameters investigated included (i). incidence of (PA or NM PA or M PA)/respiratory specimen, (ii). relative culture density of PA [NM PA or M PA]/respiratory specimen, (iii). antibiotic susceptibility.

The rate of isolation of PA was defined as frequency of PA isolated from sputum and was presented as rate/respiratory specimen. The density of PA isolated from respiratory specimen was quantitatively recorded by taking the laboratory semi-quantitative result (i.e. none, +, ++, +++) and converting these values into a fully quantitative value, ranging from 0, 1, 2 and 3, respectively, and expressed per respiratory specimen.

Antibiotic susceptibility was defined by employment of the Relative Resistance Index (RRI)[3] of PA isolates, within the following classes of antibiotics (agents analysed): aminoglycosides (gentamicin, tobramycin, amikacin); β-lactams (temocillin, ceftazidime, piperacillin/tazobactam, aztreonam), carbapenems (imipenem, meropenem), polymyxin (colistin) and fluoroquinolone (ciprofloxacin). Relative Resistance Index (RRI) values were calculated for each NM-PA and M-PA isolate in each patient, at each visit to the CF Unit, as either an in-patient or out-patient. Antibiotic susceptibility was routinely recorded as sensitive (S), intermediate (M) or resistant (R). Subsequently, for the purposes of this analysis, RRI values were assigned, as follows: a value of 1 for sensitivity, 2 is intermediate/moderate resistance and 3 for resistant.

Additionally, the number of antibiotic courses, both oral and intravenous (IV), were analysed and expressed as the mean courses of antibiotics given per month over the two years prior to commencement of ivacaftor therapy and the two years after commencement of ivacaftor therapy.

Statistical analysis

Student t-tests were used to compare pre-ivacaftor and post-ivacaftor microbiology findings. Where unpaired t-tests were employed, F values were calculated to determine equal or unequal variance. P values and confidence intervals (CIs) were two-sided, where $p \leq 0.05$ (5%) was deemed significant. Error bars were calculated as ± standard error of the mean (SEM).

Results

Antibiotic therapy

There was no significant difference between the number of oral antibiotic courses/month before and after IVA therapy (0.1 v 0.07; p=0.41) (Figure 1). There was a significant reduction in the number of iv antibiotic courses/month, following IVA therapy (0.15 v 0.02; p=0.0003) (Figure 2). There was no requirement for iv antibiotics in 6/15 (40%) patients, both prior to and following commencement of IVA therapy. Five of the patients who required iv antibiotics pre-IVA therapy (33.3%) subsequently did not require iv antibiotics, following commencement of IVA therapy. For the remainder of the patients, 4/15 (26.7%) required less courses of iv antibiotics following commencement of IVA therapy.

All patients who were taking nebulized anti-pseudomonal antibiotics for suppressive therapy continued to take these after commencement of IVA therapy and thus there was no change in the rate of inhaled antibiotics with the *Pseudomonas*-positive patients.

Rate & density of Pseudomonas aeruginosa isolation from respiratory specimens

Non-mucoid Pseudomonas aeruginosa

Eleven patients from the total 15 patients included in this study were culture-positive for NM-PA, prior to commencement of IVA therapy. Overall at a population level, there was no significant difference in either the rate (p=0.90) or density (p=0.07) of NM-PA, following commencement of IVA therapy (Table 1). When examined statistically, at individual patient level, the density of NM-PA was significantly lower (before v after commencement IVA therapy) in one patient (p=0.02), even though this patient had a constant rate of NM-PA isolation (Figure 3). One patient acquired an NM-PA after commencement of IVA therapy, but this isolate appeared to be transient, as it was only reported in a single specimen, whereby the remaining six specimens were negative (data not shown). The other

three patients remained NM-PA free.

Mucoid Pseudomonas aeruginosa

Nine patients in this study were culture-positive for M-PA, prior to commencement of IVA therapy. Overall, at a population level, there was a significant lowering in both the rate (p=0.02) and density (p=0.006) of M-PA, following commencement of IVA therapy (Table 1 & Figure 4). Interestingly, a reduced density of M-PA isolation was statistically observed in three of the nine patients (33.3%) and additionally two patients became negative for M-PA after commencement of IVA therapy. None of the six patients previously negative for M-PA prior to IVA therapy gained M-PA, after commencement of therapy.

Other microorganisms

There was no significant difference in both the rate (p=0.37) and density (p=0.43) of *Staphylococcus aureus* in patients (n=7), before v after commencement of IVA therapy. Additionally, in one patient, there was no significant difference in the rate or density of *Burkholderia cenocepacia*, before v after commencement of IVA therapy. For other non-*Pseudomonas* organisms, Table 2 details a qualitative comparison of microorganisms cultured in patients before and after commencement of IVA therapy.

Antibiotic susceptibility in PA isolates

The Relative Resistance Index [RRI][3] was employed as a semi-quantitative method to determine the susceptibility of NM-PA and M-PA. In total, 366 isolates of PA were analysed for antibiotic susceptibility against 11 antibiotics, including 220 NM-PA (n=109 & n=111 before and after commencement of IVA therapy, respectively), as well as 146 M-PA (n=77 & n=69 before and after commencement of IVA therapy, respectively). This equated to approximately 9-10 PA isolates per patient before, as well as after commencement of IVA therapy.

Figure 5a shows antibiotic susceptibility, as expressed as RRI, for total PA (NM-PA + M-PA) against

11 antibiotics within four antibiotic classes. Figure 5b shows antibiotic susceptibility, as expressed as RRI, for NM-PA and Figure 5c for M-PA.

There were no statistically significant differences in antibiotic susceptibility for the aminoglycosides, polymyxin (colistin) or fluoroquinolone (ciprofloxacin) in NM-PA and M-PA (Figure 5a-c). Whilst Figure 5a-c shows a general lowering of the RRI value, this was only statistically significant in one class of antibiotics, namely certain β-lactam antibiotics, before and after commencement of IVA therapy. For total PA, there is a significant increase in susceptibility relating to ceftazidime ($p=0.002$), tazocin ($p=0.004$), azteonam ($p=0.045$) and meropenem ($p=0.005$). Regarding NM-PA, three β-lactams showed a significant increase in susceptibility, namely ceftazidime ($p=0.03$), tazocin ($p=0.002$) and meropenem ($p=0.02$), whilst only ceftazidime showed a significant increase in susceptibility ($p=0.04$), with M-PA (see Figure 5a-c).

Discussion

With any novel non-antimicrobial pharmacological intervention in CF, it is important to carefully monitor the microbiological status of patients on therapy, in order to ensure that the intervention is not having a deleterious effect on the patients' microbiological parameters, which could potentially manifest in clinical deterioration. In this observational study, our objective was to retrospectively examine the microbiology of CF patients, who had received IVA therapy. This was achieved by examining microbiological data routinely gathered as part of the patient CF care pathway, from two years proceeding initiation of IVA therapy and continuing to approximately two years after commencement of IVA.

Overall, there was a significant reduction in the requirement for iv antibiotic courses when patients commenced IVA therapy ($p= 0.0003$) (Figure 2). Of the nine patients who required iv antibiotics, prior to the commencement of IVA therapy, five of these did not require any iv antibiotics, whilst on IVA therapy and the remaining four patients required a reduced number of iv antibiotic courses. We did not observe any patient, who did not routinely receive iv antibiotics subsequently requiring iv antibiotics whilst on IVA therapy.

In contrast, there was no statistical difference in requirement for oral antibiotics before versus after commencement of IVA therapy ($p=0.41$). Nine patients from the 15 patients examined in this study required oral antibiotics, prior to commencement of IVA therapy and seven of these patients continued to require oral antibiotics whilst on IVA therapy. Following commencement of therapy, there were two patients who did not require any oral antibiotics and one patient who did require oral antibiotics on therapy, who did not require oral antibiotics before commencement of IVA.

Similarly, in a recent study from the Czech Republic, a 21% reduction on antibiotic therapy (per patient-year) was reported ($p<0.001$), however it is not clear whether this therapy included oral, ivs or

both.[4] In our study we observed an overall significant reduction (86.7%) in the number of iv antibiotic courses per month and a 20% reduction in the number of oral antibiotic courses per month, although not statistically significant.

The question does however remain from our study, as to why iv antibiotic usage went down, whilst oral usage remained unaltered. One possible explanation is a lower clinical threshold for starting an oral antibiotic in healthier patients versus that for commencement of an iv antibiotic in sicker patients. Consequently, oral antibiotics may be an interesting first choice for examining antimicrobial stewardship within cystic fibrosis.

There was no significance difference in either the rate of isolation or density of NM-PA after commencement of IVA therapy. This was in contrast to M-PA, where there was a significant reduction in both the rate of isolation and density. To date, there have been no other reports which have divided total PA into NM-PA and M-PA components, however the study by Heltshe et al. reported significant reduction in the percentage of patients with total PA ($p=0.004$) and M-PA ($p=0.05$), following 1 year post IVA therapy.[5] More recently, a further study reported that IVA caused a marked reduction in PA density, which commenced 48 hours post commencement of IVA therapy and which continued in the first year of therapy. Following this, PA density rebounded in 6/7 patients, commencing at day 210 on IVA therapy.[6]

There is a relative paucity of data describing the fate of bacterial numbers in patients commencing IVA therapy. Recently published data from the 2013 NACFC Meeting demonstrated that appropriate patients on ivacaftor had improved mucociliary clearance[7] and demonstrated that mucociliary clearance increased from $8.5 \pm 1.7\%$ at baseline to $18.7 \pm 2.3\%$ and $17.7 \pm 1.7\%$ at one and three months post-treatment, respectively ($p < 0.001$ for each comparison to baseline). Dramatic improvements in peripheral lung clearance were also demonstrated ($1.5 \pm 1.8\%$ vs.$12.2 \pm 2.0\%$, $9.1 \pm 2.4\%$ at baseline, one month and three months, respectively; $p < 0.05$ for each comparison to baseline.

Current research, which is part of the GOAL observational study, from Sagel's group in Colorado, also presented at the recent NACFC meeting.[8] Induced sputum was collected pre- and post-ivacaftor treatment in 14 subjects (age 27 ± 14 yrs; FEV1 84 ± 23% predicted; 6 females). Sputum bacterial diversity did not change significantly with treatment [Shannon Diversity: mean change (SE) 0.13 (0.14), p=0.34]. The combined relative abundance (RA) of traditional CF bacterial pathogens including *Pseudomonas, Staphylococcus, Stenotrophomonas, Achromobacter,* and *Burkholderia* trended down with treatment [mean change (SE) -13.9 (8.2), p=0.11]. *Prevotella* RA significantly increased with treatment [mean change (SE) 8.8 (3.0), p=0.01]. By qPCR, neither total bacterial load changed significantly between paired samples [mean change (SE) −0.18 (0.16) log10 gene copies/mL, p=0.28], nor did *Pseudomonas* load [mean change (SE) −0.76 (0.66) log10 gene copies/mL, p=0.27]. There were no significant changes in any sputum markers of inflammation, including neutrophil elastase activity [mean change (SE) -0.1 (0.1) log10 μg/mL, p=0.29].

On first examination of the Sagel's data,[8] it would appear that nothing significant happened to the total bacterial counts nor the PA counts, which is counter- intuitive to data of Donaldson,[7] with improved mucocillary clearance in patients on ivacaftor. The problem here is that the Sagel group measured bacterial numbers with an inappropriate methodology, namely enumeration solely via a molecular means qPCR. Where DNA is the target for qPCR determination, it will count total bacteria in the sputum specimen and consequently this will include both living, as well as dead bacterial cells. Given the persistence of DNA from living or dead bacterial cells, therefore any changes (+ or -) in culturable bacterial numbers would be effectively missed by using this methodology. The consequences of this would thus create an uncertainty as to whether or not the culturable bacteria in CF sputum in patients on ivacaftor remained constant or not.

On considering the dynamics of growth of bacteria and bacterial CF respiratory pathogens in the CF airways in patients on ivacaftor, the published data regarding bacterial numbers counted either via molecularly[6,8] or conventionally-acquired[6] does not give any indication if such numbers are present

due to (i) bacterial cells being in the stationery phase in a relative state of senescence/dormancy but which remain culturable, (ii). actively metabolising where numbers dying off equals numbers being generated de novo and (iii). have switched phenotypically from culturable sessile & planktonic vegetative cells to non-culturable persister cells. Scenarios (i) and (ii) would show no significant change in numbers, even though the physiology leading to this point is completely different. In this regard, due to the now functional ciliated escalator, bacteria need to work extremely hard to maintain their numbers constant, i.e. the Red Queen Hypothesis, as per the data of Sagel.[8]

The consequences of which modality is happening is profound. In the case of (i) above, bacterial cells are not actively dividing nor dying, therefore they will not have a high metabolic turnover and should not be as visible to the host immune system. However, in the case of (ii) above, whilst there does not appear to be much happening, in terms of changes in bacterial counts, there is an important occult metabolism taking place, which would be very visible to the host's immune system, which would drive inflammatory processes. Therefore, it is import to understand the microbiological mechanisms underpinning the fate of constant bacterial populations within the CF airways in patients on IVA.

To date, there have been no reports on the effect of IVA therapy on antibiotic susceptibility in PA. In our study, overall, there was no decrease in antibiotic susceptibility observed in PA (NM-PA and M-PA) in this study. Four classes of commonly employed antibiotics were examined, namely aminoglycosides, β-lactams, a polymyxin and a fluoroquinolone. There was no difference in susceptibility with the aminoglycoside, the polymyxin or the fluoroquinolone. It was interesting to note the increase in susceptibility with the β-lactam class of antibiotics, in particular ceftazidime, tazocin, aztreonam and meropenem. A rationale for observing a signal in only the β-lactam antibiotics remains uncertain and this is the subject of a further study, which is underway.

Whilst our study reports statistical increase with certain antibiotic susceptibilities in PA, these values

are largely of academic interest only. These data did not demonstrate marked shifts in susceptibility which altered the susceptibility designation, from sensitive, intermediate or resistant. Given this, IVA therapy should not construed in any manner in the antibiotic management of CF patients. Importantly, antibiotic susceptibility from patients on IVA therapy is not reduced and therefore IVA therapy does not appear to have a detrimental association with antimicrobial therapy.

What is new and conclusions

Overall, although the findings of this study cannot directly correlate with the effect of IVA therapy with microbiological parameters, it is of interest to note that microbiological parameters with patients on IVA therapy were not detrimentally affected. Indeed, overall, (i) the requirement for less iv antibiotic therapy, (ii) a reduction in the rate and density of M-PA, and (iii) no reduction in antibiotic susceptibility, suggests an improvement in the microbiology status of patients on IVA therapy.

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Figure & Table Titles

Figure 1: Number of oral antibiotic course per month for patients before and after commencement of IVA therapy

Figure 2: Number of intravenous antibiotic course per month for patients before and after commencement of IVA therapy

Figure 3a: Rate of non-mucoid PA isolation before and after commencement of ivacaftor (IVA) therapy in patients who were chronically colonised prior to the commencement of IVA therapy

Figure 3b: Density of non-mucoid PA isolation before and after commencement of ivacaftor (IVA) therapy in patients who were chronically colonised prior to the commencement of IVA therapy

Figure 4a: Rate of mucoid PA isolation before and after commencement of ivacaftor (IVA) therapy in patients who were chronically colonised prior to the commencement of IVA therapy

Figure 4b: Density of mucoid PA isolation before and after commencement of ivacaftor (IVA) therapy in patients who were chronically colonised prior to the commencement of IVA therapy

Figure 5: Antibiotic susceptibility, as expressed by Relative Resistance Index, against four classes of antibiotic in (a) Total *Pseudomonas aeruginosa*, (b) Non-mucoid *P. aeruginosa* and (c) Mucoid *P. aeruginosa*, isolated from patients before and after commencement of ivacaftor therapy.

(Abbreviations: IVA= ivacaftor; M= mucoid; NM= non-mucoid; PA= *Pseudomonas aeruginosa*)

Table 1: Comparison of the rate of isolation and density of non-mucoid and mucoid *Pseudomonas aeruginosa* before and after commencement of ivacaftor therapy.

Table 2: Non-*Pseudomonas* organisms cultured from CF patients' respiratory specimens before and after commencement of ivacaftor therapy